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PROSTATE CANCER TREATMENT BASED ON THE SUPPRESSION O ANGIOGENESIS AND METASTASIS

A polymeric form of fibronectin, superfibronectin (sFN), is a novel non-cytotoxic compound that has impressive anti-metastatic effects in animal experiments. It is thought to act by interfering with adherence and/or migration of tumor cells in the host. Metastases originating from human melanoma, sarcoma and carcinoma cells have been found to be suppressed by sFN. In this stage for the project, we proposed to determine the feasibility of using sFN as an anti-prostate cancer agent by addressing three important issues: 1) demonstration that sFN is effective in preventing the spread of established tumors; 2) lack of detailed studies regarding the toxicity of sFN; and 3) lack of standardization of the sFN polymerization process.

Fibronectin is a prototypic cell adhesion molecule whose interactions with tumor cells illustrate the importance of cell adhesion in invasion and metastasis. Fibronectin was the first adhesion protein shown to be functionally altered in malignant cells (reviewed in Hynes, 1990; Ruoslahti 1996); tumor cells tend to have little or none of the fibronectin matrix that normal cells deposit around themselves in abundance. The matrix formation and adhesion of cells to fibronectin (and to many other adhesion proteins) depend on receptors that belong to the integrin family of cell surface proteins (reviewed in Ruoslahti, 1991). The principal fibronectin receptor, integrin $\alpha5\beta1$, is often down-regulated in malignant cells (Plantefaber and Hynes, 1989). Restoration of the fibronectin matrix and/or its receptor suppresses cell proliferation, migration and tumorigenicity (Giancotti and Ruoslahti, 1990; Morla et al., 1994). Thus, a fibronectin network around a cell has a restraining effect. Certain cell-cell adhesion molecules, E-cadherin in particular, have similar effects (reviewed in Gumbiner, 1996).

Adhesion to fibronectin and other matrix proteins is critical for migration, invasion and metastasis. This can be demonstrated with synthetic peptides that contain the RGD cell attachment sequence and that inhibit the interaction of cellular integrins with fibronectin and some other RGD-containing matrix molecules. These peptides are capable of inhibiting cell migration, tumor cell invasion through matrices and tissues in vitro, and block both experimental and spontaneous tumor metastasis in animals (reviewed in Ruoslahti, 1996). Unfortunately, the RGD peptides have not shown sufficient activity to have stimulated further development as anti-metastatic agents.

More recently, specific RGD peptides have been used as anti-tumor agents. Several lines of evidence have implicated the integrins $\alpha v\beta3$ and $\alpha v\beta5$ in the angiogenic process. It has been shown that αv integrins are selectively expressed in angiogenic vasculature but not selectively expressed in normal vasculature (Brooks et al., 1994; Drake et al., 1995; Clark et al., 1996; Pasqualini et al., 1997; Arap et al., 1998). Moreover, αv integrin antagonists have been shown to block the growth of neovessels (Brooks et al., 1994a, 1994b, 1995, 1997; Hammes et al., 1996); in these experiments, endothelial cell apoptosis was identified as the explanation for the

inhibition of angiogenesis and consequently tumor growth (Brooks *et al.*, 1994a, b, 1995; Varner *et al.*, 1995).

Superfibronectin (sFN) provides an effective way of utilizing the anti-cancer potential of the fibronectin system. sFN seems to be endowed with both of the anti-tumor effects of the fibronectin system; it inhibits cell migration in cultures of attached cells and blocks both adhesion and migration of suspended cells (Morla *et al.*, 1994; Pasqualini *et al.*, 1996). *In vivo* sFN displays an impressive anti-metastatic activity (Pasqualini *et al.*, 1996).

sFN is generated from soluble fibronectin by treatment with a small recombinant fragment derived from the first type III repeat of the fibronectin molecule (Morla *et al.*, 1994). This fragment, III_1-C, appears to act by interfering with the intramolecular binding interactions that keep fibronectin in its soluble configuration (Morla *et al.*, 1992); once those interactions are disrupted, the molecule undergoes self-assembly into fibrils. Alternatively, III-1C may change the conformation of the fibronectin molecule in such a way that cryptic fibronectin-fibronectin binding sites capable of driving fibril assembly are exposed (Hocking *et al.*, 1996). The polymeric fibronectin that results from the III_1-C treatment is 10-fold more adhesive to cells than fibronectin insolubilized directly from solution, hence this material is referred to as sFN (Morla *et al.*, 1994).

Our results show that sFN inhibits tumor metastasis, and in some cases, primary tumor growth *in vivo*. Treatment of various kinds of human tumor cells with sFN prior to intravenous or subcutaneous injection into immunodeficient mice blocks subsequent tumor formation (Pasqualini *et al.*, 1996). Most importantly, sFN can suppress metastasis when administered systematically. Results documented in the Results section show that prostate cancers are responsive to sFN and to III-1C in experimental models using subcutaneous or intraperitoneal administration of sFN. Most importantly, sFN treatment improved the survival of prostate cancer-bearing mice.

Existing chemotherapy cancer treatments are less than optimal in metastatic disease, which is usually lethal. Clearly, an agent capable of reducing the dissemination of cancer would be valuable. The efficacy of sFN as an anti-metastatic agent has been shown in animal experiments. The results suggest that sFN is a totally new nontoxic agent with a novel mode of action that is not likely to interfere with or be affected by other treatments. These properties provide a strong motivation to study the suitability of sFN as an adjunct treatment modality in the clinic. The aim of the work proposed in this application was to perform pre-clinical studies that will prepare the groundwork for the introduction of sFN into clinical trials.

Our ideas concerning the potential of sFN for translational trials in humans, particularly in regard to prostate cancer patients, have received support from Dr. Edward Sausville, from the Developmental Therapeutics Program at the Department of Health and Human Services. We have made sFN and the III-1C fragment available to Dr. Sausville's group. They are in the

process of confirming key aspects of our animal data. Assuming that the results are favorable, they will then consider conducting clinical trials on sFN.

sFN is a Viable Therapeutic Agent

We have performed extensive preclinical studies in order to develop sFN into a useful anti-prostate cancer therapeutic agent. Several relevant issues are addressed below:

(i) sFN is transported from the peritoneal cavity into the circulation: We have demonstrated that sFN is absorbed, and it has anti-metastatic systemic effects if administered IP (Pasqualini *et al.*, 1996).

(ii) Immunogenicity of sFN: We have immunized immunocompetent mice with sFN prepared with human fibronectin and human III1C. We were unable to find any sFN-specific reactivity in the resultant antisera, only the usual response to human fibronectin. This result argues against sFN having additional unique immunogenic properties. We have prepared the reagents for the generation of mouse sFN and have tested that in mice for immunogenicity. In this proposal, we plan to study the therapeutic effects of sFN in nude mice and in C57BL/6 mice, so that issues related to anti-tumor immune responses (however unlikely) can be studied. In addition, we have no evidence that sFN would promote inflammation. On the contrary, we have found that it also suppresses the activity of ß2 integrins in leukocytes (Koivunen *et al.* manuscript in preparation).

(iii) Degradation of sFN: Our previous studies provide data on the half-life of sFN in mice after IP administration (Pasqualini *et al.*, 1996). Those data served as the basis for our treatment schedules.

(iv) Safety, characterization and standardization of sFN: The maximum tolerated dose (MTD) of sFN has not been reached. Solubility and the volume of fluid that can be given to a mouse are the limitations. Even at 30 times the standard treatment dose, no acute toxicity was observed. Chronic administration of the therapeutic doses of sFN (100 µg/mouse, three times a week for 4 months) has revealed no obvious toxicity. We have also performed histological analysis of multiple organs from animals treated with escalating doses of sFN and have not observed acute or chronic organ damage. We are in the process of extending these studies to investigate the possible deposition of sFN in the tissues, as evaluated by immunostaining with anti-human fibronectin monoclonal antibodies. To link eventual pathological changes to sFN deposition and to investigate possible organellar alterations, we plan to perform transmission electron microscopic studies. sFN is unlikely to affect normal circulating cells and tissues because only tumor cells and angiogenic endothelial cells express activated integrins.

Although fibronectin has been previously approved for clinical trials, sFN is made by mixing monomeric FN and a simple recombinant protein. The task, therefore, is to define the combination and the polymerization protocol. We have established a few tests to characterize and standardize the production of the sFN polymer. Initially, we have used a technique based on measuring turbidity by light scattering. This test allows us to monitor the polymerization process in real time. As the turbidity reaches a plateau, it is possible to select a point at which a

maximal turbidity has been reached and will remain constant for at least several minutes. The light scattering test is used to assess the effect of time and different concentrations of fibronectin and the polymer-inducing fragment III1C on the polymerization. Different batches of fibronectin and III1C were tested to determine inter-batch variations. The results allowed us to select the optimal parameters for sFN polymerization. A similar degree of light scattering was used to normalize different sFN preparations. Our preliminary results suggest that equal turbidity, achieved by adjusting the concentration of the III1C fragment, gives equally active sFN when different batches of fibronectin are used. More recently, we have developed an assay based on OD600 readings. It only requires microtiter plates and an ELISA reader. The assay is highly reproducible. Standardization of sFN production is fast and reliable. Most importantly, the polymerization profile detected in this assay can be correlated with biological activity of the polymer *in vitro* and *in vivo*, as tested in adhesion assays and in experimental metastasis experiments using melanoma cells injected intravenously (see below).

(v) Appropriate controls in the sFN studies *in vivo*: sFN works by a unique mechanism of action that is not based on cytotoxicity. At this point, there are no other anti-ovarian cancer agents available for comparison. We have not performed studies using chemotherapy drugs as positive controls because those would be unlikely to be informative. Therefore, the main issue is to prove that sFN is effective. Our *in vivo* experiments always include extensive controls: each of the components of sFN, fibronectin and the III1C fragment, alone and "mock" sFN, which is the mixture of fibronectin and an inactive analogous recombinant fragment.

Our oncologist consultants have all indicated that they are in agreement with our focus on prostate cancer treatment as an opportunity to gather data within a reasonably fast time scale. Using the III-1C fragment alone is also an appealing possibility. Our preliminary results indicate that there is potential for the use of III-1C as an anti-prostate cancer agent (see below).

RESULTS

Anti-tumor effects of sFN

Our initial *in vivo* experiments showed that tumors formed in nude (immunoincompetent) mice by human melanoma, osteosarcoma and colon cancer cells are sensitive to ex-vivo treatment with sFN. More importantly, we proceeded to demonstrate that metastatic spread of melanoma and osteosarcoma from subcutaneous tumors or from intravenously injected tumor cells could be partially or completely inhibited with intraperitoneal injections of sFN. The size of the primary tumors was not affected by systemic or local sFN in the melanoma and osteosarcoma models. Fibronectin alone, the inducing fragment alone, and fibronectin mixed with a recombinant control protein that does not induce fibronectin fibril formation, had no effect on metastases or primary tumors in the melanoma and osteosarcoma models (Pasqualini *et al.*, 1996).

More recently we have tested sFN using multiple prostate carcinoma cell lines. When cells were injected, a dramatic reduction in the tumor burden was seen in the animals that received sFN

injections 15 minutes after the tumor cells were inoculated, whereas the animals that received fibronectin alone, the III1-C fragment or DMEM had widespread peritoneal foci. Survival was assessed on another experiment performed using cells treated ex vivo (before inoculation into the peritoneal cavity). In this case, low or no tumor take was observed in the animals that received sFN treated cells. After 3 months, all mice in the control group (the ones that received DMEM treated cells) were dead. The mice that received sFN treated cells are still alive (5 months from the start date of the experiment). Analysis of the mice in the control groups by visual inspection and histology revealed disseminated tumor loads and parenchymal liver micrometastasis (evidence of systemic disease): extensive tumor invasion and metastasis were observed upon histological examination.

The prostate cancer models provided a suitable paradigm for a clinical trial. One of the advantages is that the tumor treatment can be performed locally, which should reduce the amount of sFN. However, as we have shown previously, intraperitoneally injected sFN is transported into the blood and has a systemic effect (Pasqualini *et al.*, 1996). Thus, the systemic spread of the disease was also be curtailed by this treatment protocol. Another major advantage is that the treatment area is relatively accessible to inspection.

We have shown that sFN administration - and in some cases the III-1C fragment alone - results in therapeutic benefits even when the treatment starts after the disease is already established. In this proposal, we expanded those studies and evaluated the anti-prostate cancer effects of sFN and III-1 in orthotopic models.

This project focused on the search for strategies that would interfere with prostate cancer metastatic spread. In the context of prostate cancer, preventing distant spread of the disease would bring significant progress and improve the chances of cure in patients diagnosed with early-stage localized tumors. The physical associations involved in cell-ECM interactions rely on an assembly of multimeric ligands, which no longer occur outside the context of normal cells. We have explored this system in attempt to block angiogenesis and metastasis. Tumor cells and activated endothelial cells in angiogenic vasculature often express different integrins at high activation states. Inhibition of ECM interactions in the context of such receptors by an artificial form or matrix produced in vitro (polymeric fibronectin or superfibronectin) has shown anti-tumor effects when administered to tumor-bearing mice. In comparison to other approaches, this strategy has a number of advantages. The work we have performed so far using animal models of prostate cancer indicate that (i) It is possible to interfere with adhesive interactions that are important in metastasis by treating animals with sFN (ii) It is possible to develop a non toxic and effective compound based on the anti-metastatic and anti angiogenic activity of the fibronectin polymer. sFN is generated by mixing purified fibronectin with III-1C, a fragment derived from the first type III repeat within the molecule. It can be easily produced in bacteria at high purity. Polymeric fibronectin is highly adhesive, provided that cells express activated integrins. When exposed to superfibronectin, tumor cells and endothelial cells undergoing angiogenesis are unable to function. The "coating matrix" exerts a restrictive effect. Published results show that sFN inhibits tumor metastasis and tumor growth *in vivo* (Pasqualini *et al.*, 1996). We now have

shown that prostate cancers are responsive to sFN and to III-1C in experimental models using subcutaneous or intraperitoneal administration of sFN. Our studies were aimed at developing sFN into an anti-prostate cancer therapeutic agent. The polymer has interesting features. It may indeed become a viable therapeutic agent in the treatment of cancer patients.

Our preliminary results indicate that there is potential for the use of the fibronectin polymer and the III-1C fragment alone as anti-prostate cancer agents. These effects may be extended to bone metastasis as well, the major complication in prostate cancer. We have also generated evidence that specific inhibition of metalloprotease 2 and 9 leads to tumor regression and metastasis inhibition in vivo. Thus, we plan to develop a strategy that combines therapy with both sFN and a specific MMP inhibitor to treat prostate cancer.

PROPOSAL BODY: The task originally approved for this proposal was to test sFN against primary and metastatic prostate cancer in a transgenic mouse model.

We have used a transgenic model of prostate carcinoma to determine the effect of sFN during the malignant progression of the disease (from the preneoplastic phase to the growth of overt tumors to the development of spontaneous metastasis). This approach is suitable for the study of prostate cancer in a natural microenvironment. The antiangiogenic and the antimetastatic properties of sFN can be evaluated. In the following pages we report on the progress made during the past year.

Tumors formed in nude (immunoincompetent) mice by human melanoma, osteosarcoma and colon cancer cells are sensitive to ex-vivo treatment with sFN. Metastatic spread of melanoma and osteosarcoma from subcutaneous tumors or from intravenously injected tumor cells was inhibited with intraperitoneal injections of sFN. Fibronectin alone, the inducing fragment alone, and fibronectin mixed with a recombinant control protein that does not induce fibronectin fibril formation, had no effect on metastases or primary tumors in the melanoma and osteosarcoma models (Pasqualini et al., 1996). We have made progress in validating the sFN polymer as a therapy for prostate cancer by testing it in experiments involving prostate carcinoma animal models. (i) Prostate cancer cells bind strongly to sFN. This was determined in cell adhesion assays performed in vitro. (ii) Co-injection of sFN and tumorigenic prostate cancer cells prevents tumor formation. TRAMP mice were injected bi-weekly with the indicated treatments. Tumor growth was monitored for 5 months. Prostate cancer model, TRAMP: In this transgenic system, a prostate specific promoter from the rat probasin gene is used to target expression of the SV40 T antigen specifically to the prostate. The ability to induce prostatic disease in a systematic and predictable way *in vivo* provides a second model for the study of the activation of angiogenesis in a controlled and timed manner, yet in a different environment. This model provides additional information in that it progresses to metastasis. The disease in the TRAMP model resembles human prostate carcinoma. This model makes it possible to assess the anti-angiogenic and anti-metastatic effects of sFN.

Transgenic animals were bred and screened with the assistance of Ms. Tina Poseno, a technician in our lab that is in charge of breeding and PCR screening all our transgenic lines.

KEY RESEARCH ACCOMPLISHMENTS

● We have performed an extensive series of in vivo experiments using the Superfibronectin polymer and have been able to demonstrate therapeutic efficacy against prostate cancer.

● We have characterized the Superfibronectin polymer in detail and have standardized its production

● Our studies indicate that the Superfibronectin polymer is not toxic and is effective in prostate cancer animal models.

● We have expanded our studies to test the effect of sFN and the MMP peptide inhibitor against bone metastasis in animal models. Histopathology studies were used to determine the effects of the polymer on endothelial cells and tumor cells. The preliminary data suggest that this combined experimental design holds promise in the treatment of prostate cancer bone metastasis.

LATEST DEVELOPMENTS AND FUTURE RESEARCH BASED ON THE GOALS ACCOMPLISHED IN THIS PROPOSAL

● Sophisticated morphological studies of the vasculature of mouse prostate and prostate cancer in the TRAMP mice were performed in our laboratory in collaboration with Dr. Donald McDonald, at UCSF. We have evidence that the sFN polymer and the III-1C fragment interfere with the expansion of the vascular network that is required for tumor formation in the prostate. We plan to image such effects using similar strategies to the one employed to generate the images shown in **Figure 1**. These studies should be highly informative and will shed light into the mechanistic aspects of the anti-tumor and anti-angiogenic activity of sFN and the III-1C fragment in prostate cancer. Our preliminary data from TRAMP mice indicates that the vasculature of this tumor has certain similarities to that of some other tumors we have studied, so we can build on the experience we have gained over the past several years in studies of tumor vasculature. In terms of translating observations in mice to the human prostate, we will grow PC3 human prostate cancer cells orthotopically in nude mice, but here the tumor vasculature comes from the mouse. Therefore, we will identify specific immunohistochemical and morphological features of the vasculature of normal and cancerous prostate in mice and then apply to this information to interpreting corresponding structures in the human prostate and prostate cancer. Some of the methods used in mice (e.g., injection of fluorescent lectins or tracers, scanning electron microscopy after vascular perfusion) cannot be readily used in humans. Therefore, we will take advantage of the strengths of the mouse system as well as normal and cancerous human prostate.

REPORTABLE OUTCOMES:

"**Superfibronectin Inhibits Tumorigenesis and Metastasis in Prostate Cancer Animal Models.**" **Manuscript in preparation.**

"**Imaging normal prostate and prostate tumors using vascular targeting peptides**"
(in collaboration with the McDonald Lab, at UCSF)

CONCLUSION: Although prostate cancer is the most common cancer in men in the US, there is no universally agreed upon strategic plan for its management. Organ-confined prostate cancer can be cured by radical prostatectomy or radiation therapy. However, despite the effectiveness of various initial hormonal manipulations, non-organ-confined prostate cancer remains an incurable disease. Therapeutic options after the disease fails to respond to hormonal therapy are rather limited and, at present, no chemotherapeutic agent or regimen can be accepted as an effective standard therapy. Therefore, two critical issues in prostate cancer are (i) the management of hormone-refractory advanced and/or metastatic disease and (ii) the maximization of the possibility of cure after a radical procedure in localized disease.

We reasoned that sFN could be a useful agent against prostate cancer because it would simultaneously disrupt tumor-induced angiogenesis as well as its metastatic spread. Prostate cancer growth is highly dependent on angiogenesis and that the large majority of prostate cancer patients die of metastatic disease. It is clear from these preliminary studies that sFN administration - and in some cases the III-1C fragment alone - results in therapeutic benefits even when the treatment starts after the disease is already established. Several lines of evidence suggest that tumor growth, angiogenesis, and metastasis are dependent on matrix metalloproteinase (MMP) activity. However, the lack of inhibitors specific for the type-IV collagenase/gelatinase family of MMPs has thus far prevented the selective targeting of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) for therapeutic intervention in cancer. We have recently described the isolation of specific gelatinase inhibitors from phage display peptide libraries. We have shown that cyclic peptides containing the sequence HWGF are potent and selective inhibitors of MMP-2 and MMP-9 but not of several other MMP family members. Our prototype peptide, CTTHWGFTLC, inhibits the migration of human endothelial cells and tumor cells. Moreover, the CTTHWGFTLC-displaying phage specifically homes to angiogenic blood vessels and the synthetic peptide prevents tumor growth and invasion in animal models; treatment with CTTHWGFTLC peptide resulted in improved survival of mice bearing human tumors. These gelatinase inhibitors may prove useful in tumor targeting and anticancer therapies.

We plan to expand our studies to test the effect of sFN and the MMP peptide inhibitor against prostate cancer and bone metastasis in animal models. This is an innovative non-cytotoxic approach for treatment of prostate cancer, with potential applications for palliative treatment of non-organ-confined and for adjuvant therapy of organ-confined prostate cancer.

Appreciation for the diversity of cancer biology and the importance of vascular heterogeneity in the development of prostate cancer has just began to be realized. In our view, the results we presented delineate the uniqueness of the blood vessels in the normal prostate gland and in prostate cancer, and how therapies affecting such vessels (such as sFN and III-1C) can have significant therapeutic effects. These vascular abnormalities documented here for the very first time. We believe that our accomplishments are entirely consistent with the DOD directives of promotion of translational, organ-specific research, such as the PCRP Program.

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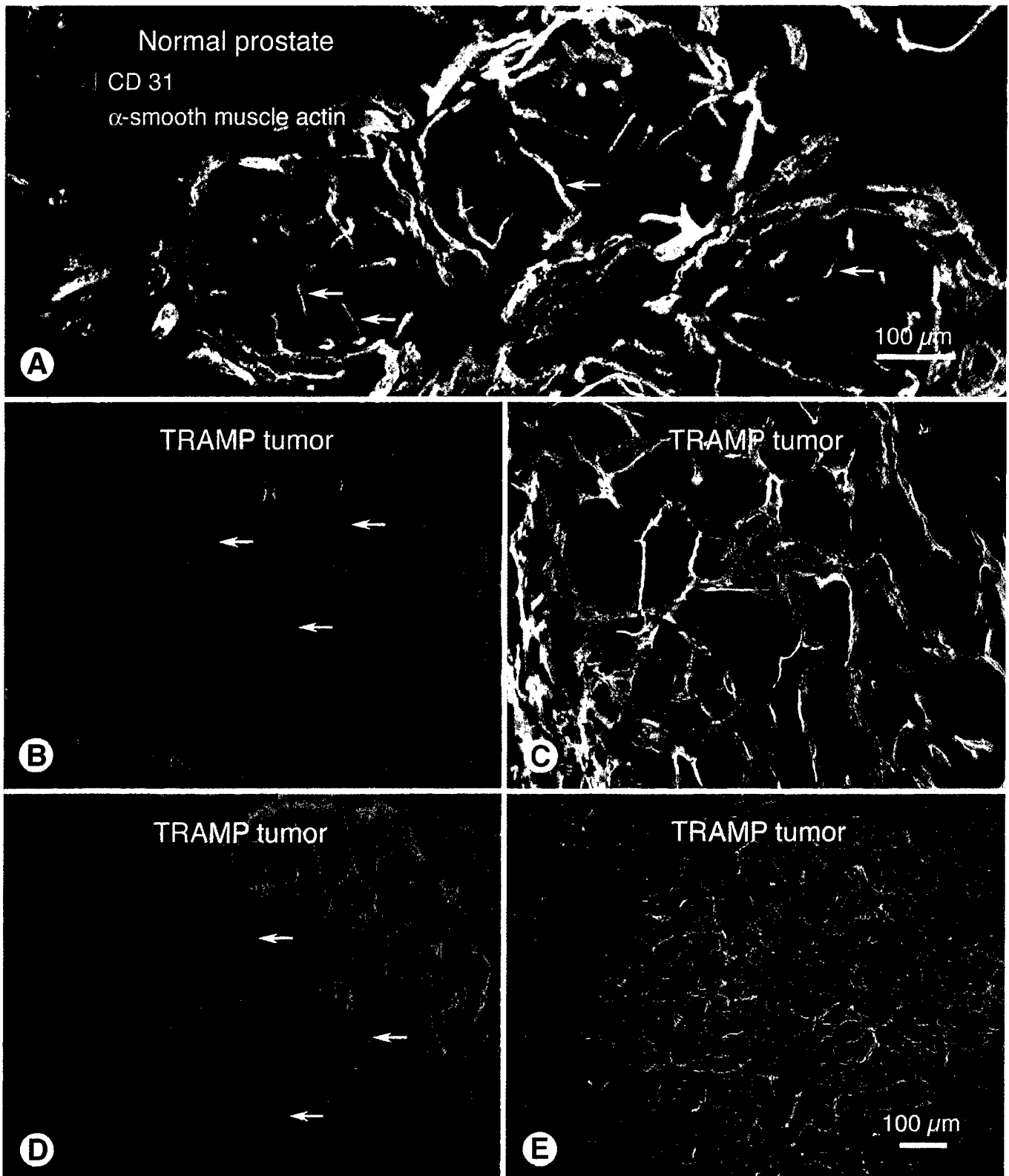
Figure 1. Vasculature of normal prostate and prostate cancer in mice. (A) In normal prostate, gland acini are surrounded by smooth muscle cell-like cells with α-smooth muscle actin immunoreactivity (red). Capillaries in normal prostate stained for CD31 immunoreactivity (arrows, green) lack α-smooth muscle actin immunoreactive pericytes. (B, C) By comparison, vessels of a large tumor in a TRAMP transgenic mouse are abnormal in size, shape, and wall structure, are interconnected by small endothelial sprouts (arrows), and have abundant α-smooth muscle actin immunoreactive cells (red). (D, E) Small CD31 immunoreactive vessels (arrows) in another TRAMP tumor are surrounded by a network of α-smooth muscle actin immunoreactive cells. Scale bar 100 μm. Bar in A applies to A; bar in E applies to B-E.